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Opposing interactions between *homothorax* and *Lobe* define the ventral eye margin of *Drosophila* eye

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ABSTRACT

Patterning in multi-cellular organisms involves progressive restriction of cell fates by generation of boundaries to divide an organ primordium into smaller fields. We have employed the *Drosophila* eye model to understand the genetic circuitry responsible for defining the boundary between the eye and the head cuticle on the ventral margin. The default state of the early eye is ventral and depends on the function of *Lobe (L)* and the Notch ligand *Serrate (Ser)*. We identified *homothorax (hth)* as a strong enhancer of the *L* mutant phenotype of loss of ventral eye. Hth is a MEIS class gene with a highly conserved Meis-Hth (MH) domain and a homeodomain (HD). Hth is known to bind Extradenticle (Exd) via its MH domain for its nuclear translocation. Loss-of-function of *hth*, a negative regulator of eye, results in ectopic ventral eye enlargements. This phenotype is complementary to the *L* mutant phenotype of loss-of-ventral eye. However, if *L* and *hth* interact during ventral eye development remains unknown. Here we show that (i) *L* acts antagonistically to *hth*, (ii) Hth is upregulated in the *L* mutant background, and (iii) MH domain of Hth is required for its genetic interaction with L, while its homeodomain is not, (iv) in *L* mutant background ventral eye suppression function of Hth involves novel MH domain-dependent factor(s), and (v) nuclear localization of Exd is not sufficient to mediate the Hth function in the *L* mutant background. Further, Exd is not a critical rate-limiting factor for the Hth function. Thus, optimum levels of L and Hth are required to define the boundary between the developing eye and head cuticle on the ventral margin.

Introduction

Axial patterning, which is crucial for the growth of multi-cellular organisms, involves the progressive restriction of cell fate by division of a homogenous group of cells into several subgroups or compartments. The selective spatio-temporal expression pattern of the cell fate selector genes results in the formation of compartments (Curtiss et al., 2002; Dahmann et al., 2011). Complex signaling events between cells of two different compartments promote proliferation and differentiation. Thus, axial patterning, which initially begins with the assignment of compartment specific fates, later contributes towards the transition of a homogeneous group of cells into a three-dimensional organ.

The adult eye of *Drosophila* develops from an epithelial bi-layer called the eye-antennal imaginal disc (Ready et al., 1976; Wolff and Ready, 1993). The embryonic eye-antennal primordium is a complex disc and is composed of cells derived from several head segments (Younossi-Hartenstein and Hartenstein, 1993). The eye-antennal disc grows and divides into eye and antennal field during larval development (Kenyon et al., 2003; Kumar and Moses, 2001). The developing eye imaginal disc comprises of two different layers viz., the peripodial membrane (PM) and the disc proper (DP). The DP gives rise to the *Drosophila* retina whereas the PM forms the head cuticle surrounding the eye (Atkins and Mardon, 2009; Cho et al., 2000; Kumar, 2011). Strict genetic regulation decides the size of the eye and its surrounding head cuticle, and this leads to the generation of the eye field boundary.

The *Drosophila* adult eye is a highly precise hexagonal array of ~800 ommatidial clusters or unit eyes. Each ommatidium has a honeycomb like hexagonal organization and comprises of eight photoreceptor

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neurons that are assembled in an asymmetrical trapezoidal pattern (Wolff and Ready, 1993). The ommatidial clusters are arranged in two chiral forms, which are arranged in mirror image symmetry along the Dorso-Ventral (DV) midline called the equator. The eye-antennal imaginal primordium begins from a group of ~20 progenitor cells (Garcia-Bellido and Merriam, 1969; Poulson, 1950; Yamamoto, 1996). The border between the dorsal and the ventral eye compartment, the equator, is the site of activation of Notch (N) signaling, which is responsible for cell proliferation and differentiation in the developing eye disc (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Singh et al., 2005b).

The early eye primordium has a default ventral fate, which depends on the function of *L* and *Ser* (Oros et al., 2010; Singh et al., 2005b; Singh and Choi, 2003). Later, with the onset of expression of the GATA family zinc finger transcription factor *pannier (pnr)*, the dorsal fate is established over the default ventral eye fate in a subset of eye primordium cells (Dominguez and Casares, 2005; Oros et al., 2010; Singh et al., 2005b; Singh and Choi, 2003). Pnr acts upstream of Wingless (Wg), which in turn induces the expression of members of *Iroquois Complex (Iro-C)* genes viz., *araucan (ara)*, *caupolican (caup)* and *mirror (mirr)*. *Iro-C* genes act downstream of *pnr* and *wg*, and are expressed in the dorsal half of the developing eye imaginal disc. *Iro-C* genes are required for assigning dorsal eye fate and triggering Notch pathway in the DV boundary of the eye (Cavodeassi et al., 1999; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Singh et al., 2005b). *pnr* is expressed in the peripodial membrane on the dorsal margin of the eye disc (Oros et al., 2010; Pichaud and Casares, 2000). Recent studies have demonstrated that Pnr suppresses the eye fate and thereby defines the boundary between the head cuticle and the dorsal margin of the developing eye field (Oros et al., 2010). Since Pnr is expressed only in the dorsal eye margin therefore, *pnr* is not involved in genetic mechanism regulating the developing eye field boundary on the ventral margin. Thus, the genetic mechanism regulating the boundary of eye field on the ventral margin remains unclear.

In the ventral eye, the loss-of-function of *homothorax (hth)*, results in eye enlargements or ectopic eyes (Pai et al., 1998; Pichaud and Casares, 2000). *hth* encodes a homeodomain transcription factor of the three-amino-acid extension loop (TALE) subfamily with extensive amino acid identity to the murine proto-oncogene *Meis1* (Moskow et al., 1995; Rieckhof et al., 1997). Even though *hth* is expressed uniformly anterior to the furrow both in the dorsal and the ventral half of the eye, loss-of-function clones exhibit enlargements only in the ventral half of the eye whereas the clones in the dorsal half of the eye do not exhibit any eye phenotypes (Pai et al., 1998; Pichaud and Casares, 2000). However, misexpression of *hth* suppresses the eye irrespective of the dorsal or the ventral fate. Thus, *hth* is known to act as the negative regulator of eye development (Pai et al., 1998). Hth has a nuclear localization signal (NLS) and two conserved domains: the N terminal evolutionarily conserved MH domain (for Meis and Hth), and a C-terminal region including the homeodomain (HD) (Jaw et al., 2000; Noro et al., 2006; Pai et al., 1998; Rieckhof et al., 1997; Ryoo et al., 1999). Alternative splicing is known to provide additional complexity to the genes encoding the transcription factors (Glazov et al., 2005; Noro et al., 2006). Alternative splicing at *hth* locus results in generation of different Hth isoforms. It has been reported that seven different mRNA are transcribed from *hth* genomic region. These transcripts can be classified into three classes of one long and two short transcripts (Noro et al., 2006; Salvany et al., 2009). In our study, we employed two Hth protein isoforms: a full length/long protein (Hth-FL) containing both MH and HD domain and a second short form that lacks the HD (HD-less) (Glazov et al., 2005; Noro et al., 2006).

In *Drosophila*, the sub-cellular localization of another homeoprotein Extradenticle (Exd) is tightly regulated by Hth. In the absence of Hth, Exd is localized in the cytoplasm, while in the presence of Hth, Exd forms a heterodimer with Hth through its MH domain and translocates into the nucleus to regulate transcription (Abu-Shaar et al., 1999; Aspland and White, 1997; Jaw et al., 2000; Stevens and Mann, 2007). Hth and Exd are also involved in forming a heterodimer with other HOX proteins that alter their DNA binding specificity in the nucleus (Mann, 1995; McGinnis and Krumlauf, 1992). Hth and Exd are involved in a direct protein–protein interaction that is mediated through the N-terminal MH domain. In the eye, Exd is uniformly expressed. However, Exd is nuclear only in the domains where Hth is expressed (Mann and Abu-Shaar, 1996; Rieckhof et al., 1997; Stevens and Mann, 2007), which is the region of the eye disc that develops into the head cuticle surrounding the compound eye (Pai et al., 1998). Thus, Hth and Exd promote head specific fate.

Here we address how *L*, a gene required for ventral eye development and survival, interacts with *hth* to control ventral eye growth. We found that antagonistic interaction between *L* and *hth* is responsible for defining the size and boundary of the eye field on the ventral margin. Further, L and Hth interaction is mediated by a novel mechanism that requires the MH domain of Hth but does not require Exd.

Materials and methods

Fly stocks used are described in Flybase (http://flybase.bio.indiana.edu). We used the following *L* mutants in this study: $L^{rev6\text{-}3}$ *FRT42D/CyO*, L^{2}/*CyO*, L^{si} (Chern and Choi, 2002; Singh and Choi, 2003), and UAS-*L RNA*i (available at VDRC, http://stockcenter.vdrc.at/control/main). $L^{rev6\text{-}3}$ is a null allele of L (Chern and Choi, 2002), L^{2} is a dominant negative allele (Singh et al., 2005a); and L^{si} is a hypomorph (Chern and Choi, 2002). The *hth* alleles used in this study are: hth^{P2}, $hth^{100\text{-}1}$ and $hth^{1422\text{-}4}$ (Kurant et al., 2001; Noro et al., 2006; Pai et al., 1998). hth^{P2} is a strong hypomorph generated by P-element insertion in *hth* promoter (Pai et al., 1998). *hth* consists of 16 annotated exons. The MH and HD domains are encoded by exons 2–6 and 11–13, respectively. $Hth^{100\text{-}1}$ is predicted to encode only HD-less isoforms due to an Arg321 to opal mutation in exon 9 (Kurant et al., 1998; Noro et al., 2006). $hth^{1422\text{–}4}$ is a P-element insertion line that serves as an excellent reporter for *hth* expression in the eye imaginal disc (Pai et al., 1998; Salzberg et al., 1997).

We used the Gal4/UAS system for the targeted misexpression studies (Brand and Perrimon, 1993). We used *ey*-Gal4 (Hazelett et al., 1998) to drive expression of the transgene in the developing eye field for the gain-of-function studies (Singh et al., 2005a). Various UAS-transgenes used in this study are: UAS-EN-$HTH^{1\text{-}430}$ or UAS-EN-Hth^{ENR} a dominant negative allele of *hth*, generated by fusing the *Drosophila* EN repression domain (Han and Manley, 1993) to a truncated form of Hth (amino acids 1–430) (Inbal et al., 2001), UAS transgenes harboring the full length *hth* (*hth*-FL), and transgenes lacking either Homeodomain (ΔHD) or the Meis Homothorax domain MH (ΔMH) were used for targeted misexpression studies (Jaw et al., 2000; Ryoo et al., 1999). All Gal4/UAS crosses were done at 18 °C, 25 °C and 29 °C, unless specified, to sample different induction levels.

Genetic mosaic analysis

We employed genetic mosaic approach to generate loss-of-function clones in the eye (Xu and Rubin, 1993). For the generation of clones in the eye, we have used *ey*FLP (Newsome et al., 2000) as source of flippase. To generate mosaic clones of (i) *L* in the eye, *eyFLP; FRT42D ubi-GFP* virgins were crossed to males of L^{rev}*FRT42D/CyO*, (ii) *hth* in the eye, *eyFLP; FRT82B ubi-GFP* virgins were crossed to *y, w; FRT 82B* hth^{P2} or *FRT 82B* $hth^{100\text{-}1}$*/TM6B* males. Mutant tissue was marked by the absence of GFP reporter.

Immunohistochemistry

Eye-antennal imaginal discs were dissected from wandering third instar larvae and stained following the standard protocol (Singh et al.,

2002). Antibodies used were rat anti-Elav (1:100), mouse anti-Wg (1:50) (Developmental Studies Hybridoma Bank), rabbit anti-Dlg, anti-Hth (H. Sun and R. Mann), rabbit anti-Exd (Aspland and White, 1997; Mann and Abu-Shaar, 1996), and rabbit anti-Mirr (1:200). Secondary antibodies (Jackson Laboratories) used in this study were goat anti-rat IgG conjugated with Cy5 (1:200), donkey anti-rabbit IgG conjugated to Cy3 (1:250), donkey anti-rabbit IgG conjugated to FITC, and donkey anti-mouse IgG conjugated to Cy3 (1:200). Immunofluorescent images were analyzed using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope.

Results

hth is a modifier of L in the ventral eye

The *L* gene function is required for ventral eye development and growth (Chern and Choi, 2002; Singh and Choi, 2003). Loss-of-function of *L* results in the selective loss of ventral eye in the larval eye imaginal disc (Fig. 1D) and the adult eye (Fig. 1C) as compared to the wild-type eye (Figs. 1A, B). We have identified *hth* as a modifier of this *L* mutant eye phenotype of selective loss-of-ventral-eye. Increasing levels of *hth* gene function in the *L* mutant eye imaginal disc using gain-of-function approach ($L^2/+$; *ey>hth*), results in the enhancement of ventral eye loss to a "no-eye" phenotype as seen in the third instar larval eye imaginal disc (Fig. 1H) and the adult eye (Fig. 1G). Loss of eye fate as a result of induction of Hth (L^2/*CyO; ey>hth*) is due to eye to cuticle fate change. Thus, increasing levels of *hth* gene function enhances the *L* mutant phenotype in the eye suggesting that *hth* acts as a genetic modifier of *L* mutant.

Therefore, we explored the mechanism by which Hth modified the *L* mutant phenotype of loss-of-ventral-eye. First, we tested if loss of *L* affects *hth* expression in the ventral eye. In the developing third instar eye imaginal disc, Hth is strongly expressed anterior to the furrow, which corresponds to the region that forms the ptilinum, ocellus, head capsule, and also in the posterior and lateral margins of the eye disc (Fig. 1B). Hth is expressed in the cells of the peripodial membrane of the eye disc and weakly in the posterior region that is composed of mature photoreceptors (Bessa et al., 2002; Pai et al., 1998; Pichaud and Casares, 2000; Singh et al., 2002). Even though Hth is a transcription factor that needs to be localized in the nucleus, it is present both in the cytoplasm as well as the nucleus whereas L is located in the cytoplasm (data not shown). We found that in *L* mutant background Hth expression was upregulated (Fig. 1D; arrow). Since the majority of cells in the ventral half of the eye are lost in the $L^2/+$ mutant eye imaginal disc (Singh et al., 2006), Hth upregulation was seen only on the ventral margin (Fig. 1D, arrow). However, there is a need to verify if it is an additive effect or a real interaction since increasing levels of *hth* alone in the eye (*ey>hth)* results in the suppression of eye (Pai et al., 1998; Singh et al., 2002). Hth is known to be a negative regulator of the eye (Pai et al., 1998). In order to test the genetic interaction between *L* and *hth*, we decided to analyze their loss-of-function phenotypes in the eye.

L and hth exhibit complementary loss-of-function phenotype in eye disc

Loss-of-function clones of L^{rev} in the eye exhibit domain specific phenotype. Loss-of-function clones of *L* in the ventral eye result in the selective loss of eye fate (Figs. 2A, A′) as evident from suppression of neural marker ELAV (Fig. 2A′). However, in the dorsal eye these clones have no effect on the eye fate. Interestingly, loss-of-function clones of *hth* in the ventral eye result in eye enlargement or induction of ectopic eye (Pai et al., 1998) whereas in the dorsal eye these clones do not affect the eye fate (Figs. 2B, B′, arrow). Thus, *hth* loss-of-function clones also exhibit a dorsal–ventral constraint in their phenotypes. Given the opposing outcomes of *hth* and *L* loss-of-function on the ventral eye fate, we further explored the interaction of *L* and *hth* by testing the expression of Hth in the *L* mutant cells in the eye imaginal disc. Interestingly, both *L* and *hth* are not expressed in a domain specific manner during eye development (Bessa et al., 2002; Singh and Choi, 2003).

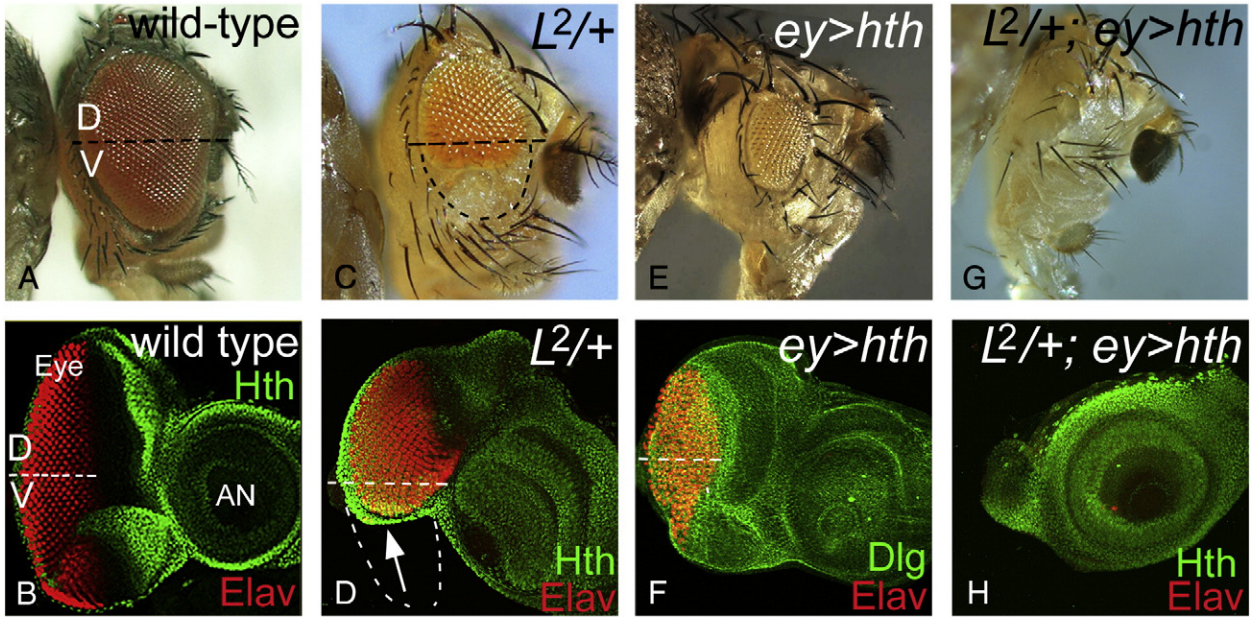

Fig. 1. *hth* acts as a modifier of the *L* mutant phenotype of preferential loss-of-ventral-eye. (A, B) Wild-type adult eye and eye imaginal disc are shown. The border between the dorsal (D) and the ventral (V) compartment of the eye marks the equator. (B) Hth (green) is expressed only anterior to the morphogenetic furrow (MF) in the eye disc, which corresponds to the adult head cuticle. Elav (red), a pan neural marker, marks the photoreceptors in the eye. (C, D) $L^2/+$ mutant exhibits preferential loss-of-ventral-eye phenotype in the eye disc (marked by the dotted line) and adult eye. (D) $L^2/+$ mutant eye disc exhibits strong induction of Hth (green) on the ventral eye margin. Since the majority of ventral eye is lost in the third instar eye disc, we can see ectopic Hth expression only on the ventral margin (arrow). (E, F) Misexpression of *hth* in the entire eye using the *ey*-GAL4 driver (*ey>hth*) suppresses the eye fate in the eye imaginal disc and the adult eye. (G, H) Misexpression of *hth* in the $L^2/+$ mutant eye background ($L^2/+$; *ey>hth*) results in strong enhancement of loss-of-ventral-eye phenotype to a "no-eye" phenotype as evident from absence of any Elav positive cells in the eye disc. Note that there is a change in eye to head cuticle fate. All images are oriented as dorsal (up), ventral (down), anterior (right), and posterior (left).

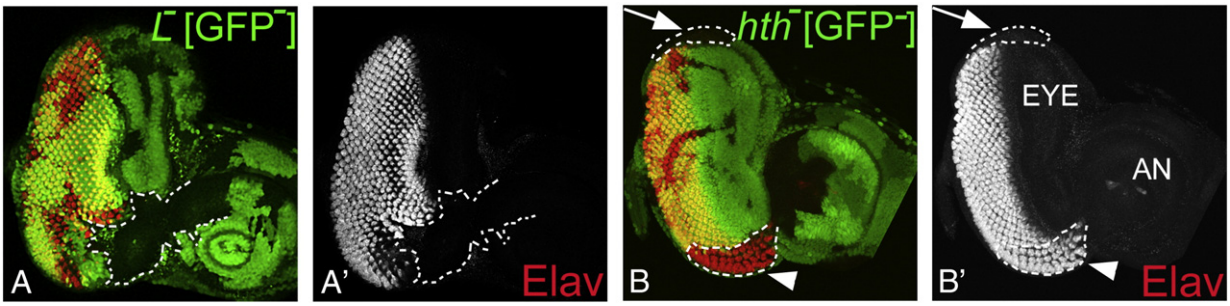

Fig. 2. Loss-of-function phenotype of *L* is complementary to *hth* in the ventral eye. (A–A′) Loss-of-function clones of L^{rev} marked by absence of the GFP reporter (clonal boundary marked by dotted line) in the ventral eye result in the (E′) suppression of eye as marked by expression of Elav, a pan neural marker whereas the clones in the dorsal eye do not affect the eye. (B, B′) Loss-of-function clones of *hth* in the eye (marked by the dotted line) show ventral eye enlargement but no effect in the dorsal.

Hth is ectopically induced in the L mutant clones

The loss-of-function clones of *L* in the ventral eye exhibit a loss of eye fate based on the absence of the pan-neural marker Elav, which marks the photoreceptor specific fate (Figs. 3B, B′–B″). In comparison to wild-type Hth expression in the eye disc (Fig. 3A), these loss-of-function clones of *L* in the ventral eye showed robust induction of Hth expression (Figs. 3B, B′ clone boundary marked by white dotted line, inset shows Hth upregulation in ventral eye clone) whereas the dorsal clones do not effect the eye fate or the Hth expression (Figs. 3C, C′, C″, clone boundary marked by white dotted line). We have counted 51 *L* loss-of-function clones. The distribution of these clones is 42 in the dorsal eye and 9 in the ventral eye. The dorsal clones did not show any effect on eye fate as well as Hth expression. The 9 ventral clones showed ectopic Hth induction and concomitant loss of eye fate. The discrepancy in the number of dorsal versus ventral clones is because of the fact that *L* mutant clones in the ventral eye do not survive (Singh et al., 2006). We further tested this interaction using an enhancer trap line where the *lacZ* reporter gene is expressed under the *hth* promoter. Because the mutant L^2 eye discs show a complete loss of the ventral eye, we tested the expression of *hth*-reporter in a hypomorphic *L* mutant L^{si}, where the heterozygous eye has an anterior nick in the eye or wild-type eye (Chern and Choi, 2002). Interestingly, this *hth* reporter showed ectopic expression in the ventral margin of the eye imaginal disc in the heterozygous *L* ($L^{si}/+$) mutant background (Fig. 3D; arrow). Next, we tested the *L* and *hth* interaction using L RNAi. Misexpression of UAS-L RNAi in the eye using *ey*-Gal4 (*ey*>*L* RNAi) resulted in a highly reduced eye field where ventral half of the eye is lost along with upregulation of Hth on the ventral eye margin (Fig. 3E). Thus, any loss of eye fate in the *L* loss-of-function clones is associated with the induction of Hth. Interestingly, *L* and *hth* interaction seems to exhibit a domain constraint based on the restriction of their loss-of-function phenotypes only to the ventral eye even though they are expressed both in the ventral and the dorsal eye (Bessa et al., 2002; Pai et al., 1998; Singh et al., 2002).

L acts antagonistically to hth

We analyzed genetic interactions between these two genes. We found that reducing the levels of *hth* to half in the *L* mutant background ($L^2/+$; $hth^{1422\text{-}4}/+$) exhibits a partial rescue of the *L* mutant phenotype of loss-of-ventral-eye (Fig. 1C) in the eye imaginal disc (Figs. 3F, G) as well as the adult eye (Fig. 3H). We employed a dorsal fate marker, Mirr expression to show the rescue of the ventral eye (Fig. 3G). We also tested this interaction by misexpressing UAS-hth^{ENR}, the dominant negative allele of *hth* in the *L* mutant eye disc (L^2; *ey*>hth^{ENR}). The repressor form of Hth was generated by fusing the *Drosophila* EN repression domain (Han and Manley, 1993) upstream to a truncated form of Hth (amino acids 1–430; EN-Hth^{1-430}) (Inbal et al., 2001). We found that the misexpression of UAS-hth^{ENR} in *L* mutants (L^2; *ey*>hth^{ENR}) caused a significant rescue of the loss of ventral eye phenotype in the eye imaginal disc (Figs. 3I, J) as well as the adult eye (Fig. 3K). We also tested whether the rescue was due to growth of the ventral eye by using Mirr expression as a marker for the dorsal fate. We found that dorsal specific expression of Mirr was restricted only to the dorsal half and there was a significant rescue of the ventral eye fate (Fig. 3J). Thus, reducing Hth levels can rescue the *L* mutant phenotype in the ventral eye. On the contrary, increasing the levels of *hth* in the *L* mutant eye imaginal disc ($L^2/+$; *ey*>*hth*) enhances the loss of ventral eye phenotype to a "no-eye" phenotype (Figs. 1G, H). There was no effect on the antennal field. Thus, a reduction or increase in the levels of *hth* in the *L* mutant eye disc has converse effects on the loss-of-ventral-eye phenotype. Our results clearly suggest that *L* genetically interacts with *hth* in the ventral eye and this interaction is antagonistic in nature (Fig. 3L).

L requires MH domain of Hth for its interaction in the eye

Since we found that *L* acts antagonistically to *hth* (Fig. 3), we next focused on identifying the domain of Hth that interacts with *L*. Hth encodes a protein with an evolutionarily conserved MH domain and a DNA binding homeodomain (Fig. 4A) (Inbal et al., 2001; Jaw et al., 2000; Ryoo et al., 1999). To test the domain specific requirement of Hth for its interaction with L, we used transgenic constructs that misexpress truncated forms of Hth to study their effect on the *L* mutant phenotype (Fig. 4A; Jaw et al., 2000; Ryoo et al., 1999). We tested individually the MH domain and the homeodomain of Hth for their requirement in interaction with *L* in the eye using the gain-of-function approach. Misexpression of ΔMH domain of *hth* in the eye (*ey*>$hth^{\Delta MH}$) does not affect the eye size (Fig. 4B) whereas misexpression of ΔHD (*ey*>$hth^{\Delta HD}$) results in suppression of the eye (Fig. 4C). In *L* mutant eye imaginal disc, overexpression of the *hth* transgene lacking only the MH domain (L^2; *ey*>$hth^{\Delta MH}$) did not affect the loss-of-ventral-eye phenotype of the *L* mutant as seen in the eye imaginal disc (Fig. 4F) as well as the adult eye (Fig. 4D). However, when we misexpressed the *hth* construct lacking the homeodomain (HD) in the *L* mutant eye background (L^2; *ey*>$hth^{\Delta HD}$), it resulted in a "no-eye" phenotype in the eye imaginal disc as well as the adult eye (Figs. 4E, G). These phenotypes are comparable to the ones seen with misexpression of the full length *hth* transgene in eye imaginal disc and the adult eye (Figs. 1G, H). These results suggest that the MH domain of Hth is crucial for its antagonistic interaction with L.

L interacts with the alternative splice variant of hth with only the MH domain

In this study we used the two different alternative spliced variants of *hth*, one with the HD domain and the other without HD (Noro et al., 2006). To address the function of MH domain *in vivo*, we utilized the $hth^{100\text{-}1}$ mutant that results in a HD-less form of Hth (Fig. 5A; Noro et al., 2006). We found that the loss of function of *hth* using the null allele results in ventral eye enlargement as

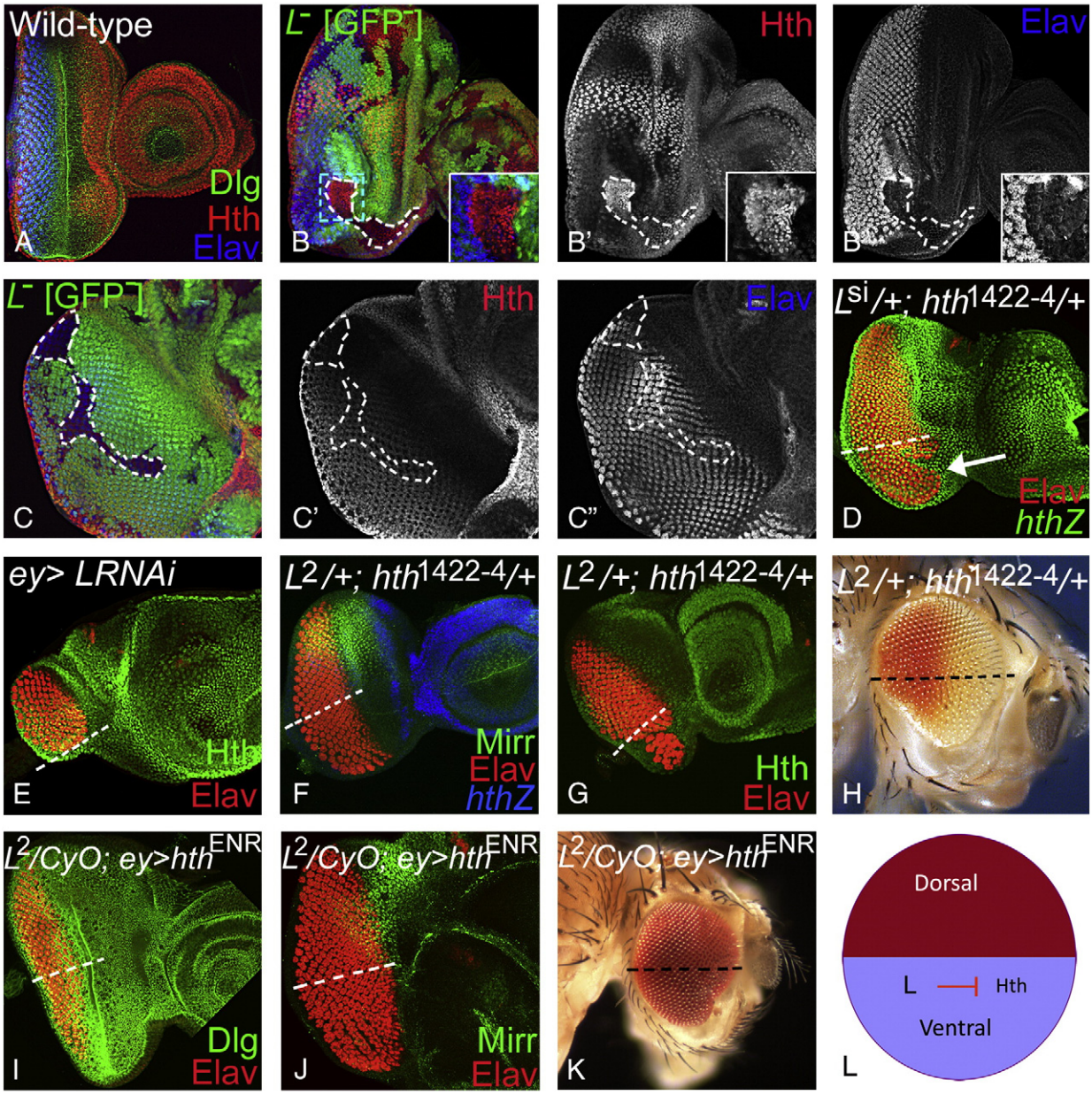

Fig. 3. *L* interacts antagonistically with *hth*. (A) Wild-type expression of Hth (red) in the eye imaginal disc. Dlg (green) marks the membrane and Elav (blue) marks the photoreceptor neuron fate. (B–B″) Loss-of-function clone of L^{rev}, which shows selective loss-of-ventral-eye fate as evident from the loss of Elav (blue) positive cells, is also accompanied with the ectopic induction of Hth (red). The insets in B–B″ show a magnified view of the ventral clone. (C–C″) Loss-of-function L^{rev} clones in the dorsal eye (clone boundary marked by the dotted line) had no effect on the eye fate and lacked any ectopic induction of Hth in the eye. (D) Misexpression of UAS-*L* RNAi (*ey*>LRNAi) results in suppression of the eye fate with ectopic induction of Hth (green) on the ventral eye margin. (E) A *lacZ* reporter under the *hth* promoter which is expressed in a specific domain anterior to the MF in the developing eye imaginal disc shows ectopic induction in the ventral eye in the $L^{si}/+$ heterozygous background marked by an arrow. (F–H) Reducing the levels of *hth* function to 50% using a null allele $hth^{1422\text{-}4}$ in the $L^2/+$ heterozygous background ($L^2/+$; $hth^{1422\text{-}4}/+$), results in the partial rescue of the loss-of-ventral-eye phenotype. (F, G) Reducing the *hth* function by dominant-negative hth^{ENR} in the *L* mutant background ($L^2/+$; *ey*>hth^{ENR}) results in the significant rescue of the loss-of-ventral-eye phenotype in the (F) eye imaginal disc and the (G) adult eye. (H) L antagonizes Hth in the ventral eye. Interestingly, this interaction does not hold true in the dorsal eye even though both L and Hth are expressed in the dorsal eye.

seen in the adult and the eye imaginal disc (Figs. 5B, C; Pai et al., 1998; Pichaud and Casares, 2000). Interestingly, when we generated loss-of-function clones of *L* in the heterozygous background of the *hth* null allele ($L^{-/-}$; $hth^{-/+}$), they did not show any suppression of the eye fate in the ventral eye (Fig. 5F). This phenotype is different from *L* loss-of-function clone phenotypes ($L^{-/-}$) of loss of ventral eye (Figs. 2A; 3A). Loss-of-function clones of $hth^{100\text{-}1}$ did not show any significant ventral eye enlargement or ectopic ventral eye in the adult (Fig. 5D) or the eye imaginal disc (Fig. 5E). However, when we generated *L* loss-of-function clones in the heterozygous background of $hth^{100\text{-}1}$ ($L^{-/-}$; $hth^{100\text{-}/+}$), these clones resulted in complete loss-of-ventral-eye (Fig. 5G) as seen in the *L* loss-of-function clones (Fig. 2A). These results further validated that the highly conserved MH domain of *hth* is crucial for its antagonistic interaction with *L*. However, the HD is dispensable for L and Hth interaction. Interestingly, the same MH domain of Hth is required for its interaction with Exd in the eye. Therefore, we tested if L interacts with Hth through Exd in the ventral eye.

L does not interact with Exd to define the ventral eye margin

Hth is known to form a heterodimer with Exd and the resultant complex moves to the nucleus to regulate transcription of the target genes (Abu-Shaar et al., 1999; Aspland and White, 1997; Jaw et al.,

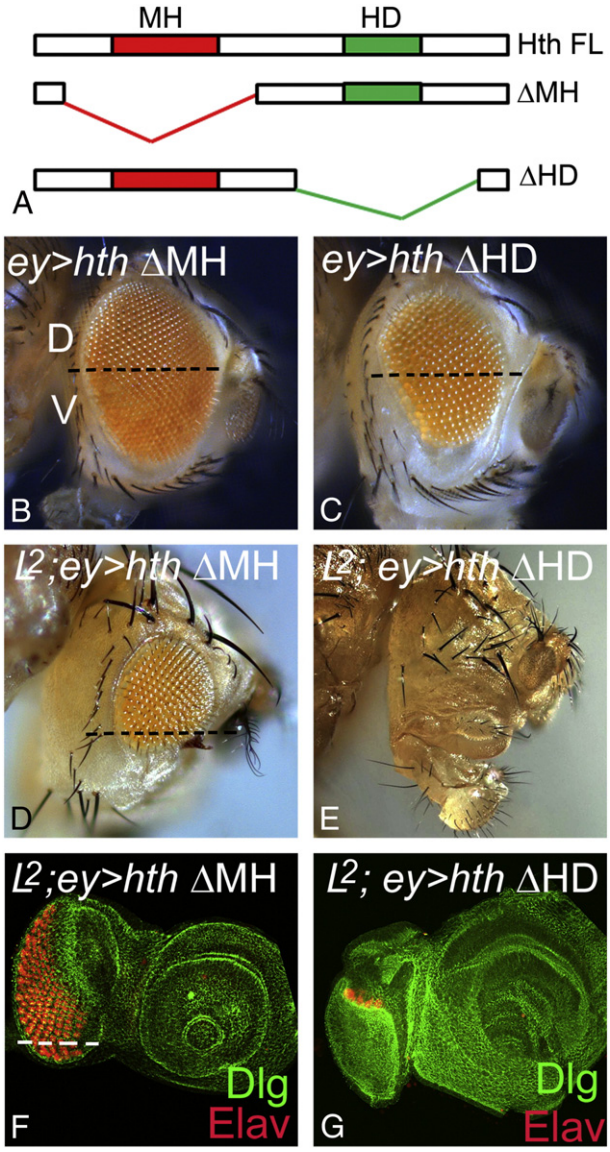

Fig. 4. L requires MH domain of Hth for its interaction. (A) Hth encodes a protein with the MH- and the homeodomain (HD). Several transgenic lines expressing truncated Hth protein were used to test the requirement of various domains of the Hth protein in its interaction with L. (B, C) Misexpression of truncated Hth where (B) MH domain is missing (*ey>hth*$^{\Delta MH}$) does not affect the eye size and (C) HD is missing (ey>*hth*$^{\Delta HD}$) results in small eye. (D, F) Misexpression of Hth$^{\Delta MH}$ (*L*^{2}*; ey>hth*$^{\Delta MH}$) in a *L* mutant background does not affect the loss-of-ventral-eye phenotype, as seen in the (D) eye imaginal disc and the (F) adult eye. (E, G) Misexpression of *hth*$^{\Delta HD}$ in the *L* mutant eye background (*L*^{2}*; ey>hth*$^{\Delta HD}$) results in a "no-eye" phenotype as seen in the case of (Figs. 1G, H) full length *hth* misexpression.

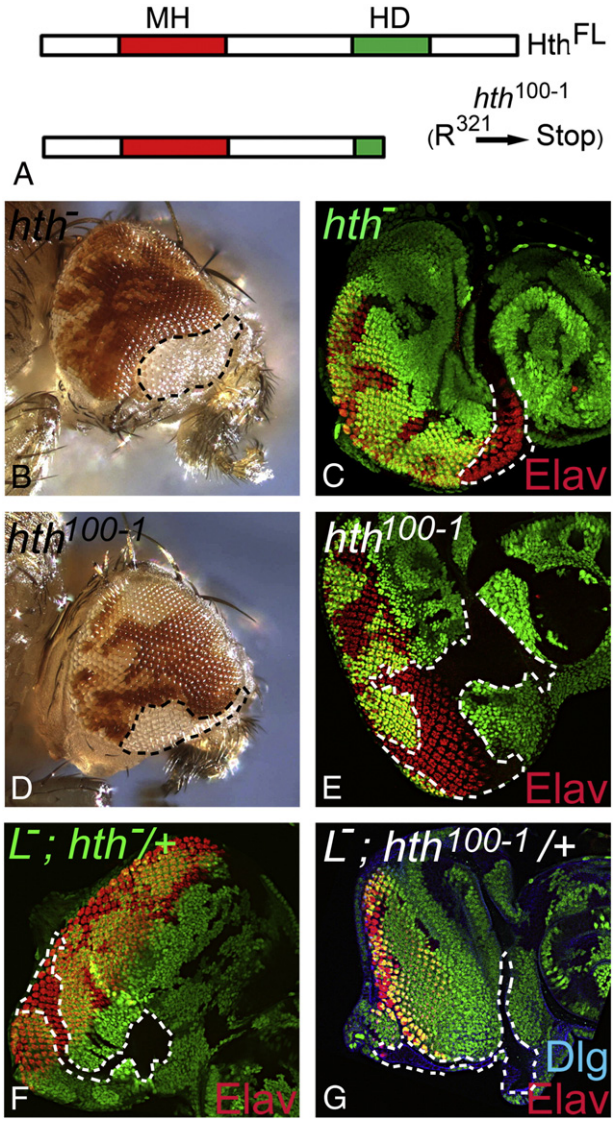

Fig. 5. L interacts antagonistically with alternative spliced variant of Hth. (A) Hth encodes a protein with the MH- and the homeodomain (HD). *hth*$^{100-1}$, an allele of *hth*, which encode a HD less isoform due to an Arg321 to opal mutation in exon 9 (Kurant et al., 2001; Noro et al., 2006). (B, C) Loss-of-function clones of null allele of *hth* (clone boundary marked by dotted line) results in ectopic ventral eye enlargement as seen in the (B) adult and (C) eye imaginal disc. (D, E) Loss-of-function clones of *hth*$^{100-1}$, which selectively eliminate the alternative spliced variant that affects only the full length Hth and not the one with only the MH domain, results in no effect on the ventral eye in (D) adult and (E) eye imaginal disc. (F) Loss-of-function of L^{rev} in the heterozygous background of null *hth* results in the rescue of the ventral eye loss. Note that the heterozygous *L* null and *hth* null show a normal eye (Singh and Choi, 2003; Singh et al., 2002). (G) Loss-of-function clone of L^{rev} in the heterozygous background of *htm*$^{100-1}$ result in the loss of ventral eye as seen in the *L* loss-of-function clones. Heterozygous *hth*$^{100-1/+}$ control exhibits a normal eye. However, it causes antenna to leg transformation as seen in *hth* loss-of-function (Casares and Mann, 1998).

2000; Rieckhof et al., 1997; Stevens and Mann, 2007). It is possible that L might prevent Hth–Exd binding in the cytoplasm. Therefore, we tested whether L–Hth interaction also requires Exd or is independent of Exd function. Exd is present in the cytoplasm in the eye imaginal disc, but Exd localization becomes nuclear only where Hth protein is present (Figs. 6A, A′, A″′). It has been shown that Exd is functional only when it is localized in the nucleus (Mann and Abu-Shaar, 1996; Rieckhof et al., 1997; Stevens and Mann, 2007). To test whether *L* interacts with *exd* in the ventral eye, we generated *L* loss-of-function clones in the eye and tested the expression of Exd. The *L* loss-of-function clones in the ventral eye showed strong ectopic nuclear localization of Exd along with a loss of Elav (Figs. 6B–B‴, inset). These results further suggest that either *L* interacts antagonistically with both *exd* and *hth* or with *hth* alone. Therefore, we tested epistatic interactions between *L* and *exd*. The rationale of the experiment was if L and Exd interact antagonistically to each other, then reducing *exd* function will rescue the *L* mutant phenotype. In the *L* mutant heterozygous background that exhibits loss-of-ventral-eye, we further reduced the *exd* gene function (*exd*^{1}*/+; L*^{2}*/+*), and found that the *L* loss-of-ventral-eye phenotype remains unaffected (Fig. 6C). Conversely, we overexpressed *exd* in the *L*^{2} mutant eye background (*L*^{2}*/+; ey>exd*) and found that the *L* mutant phenotype of loss-of-ventral-eye was not affected (Fig. 6D). We also generated *L* loss-of-function clones in an *exd* heterozygous background but found no effect on the *L* loss-of-function clone phenotype of loss-of-ventral-eye (Fig. 6E). These results suggest that *L* and *exd* may not interact with

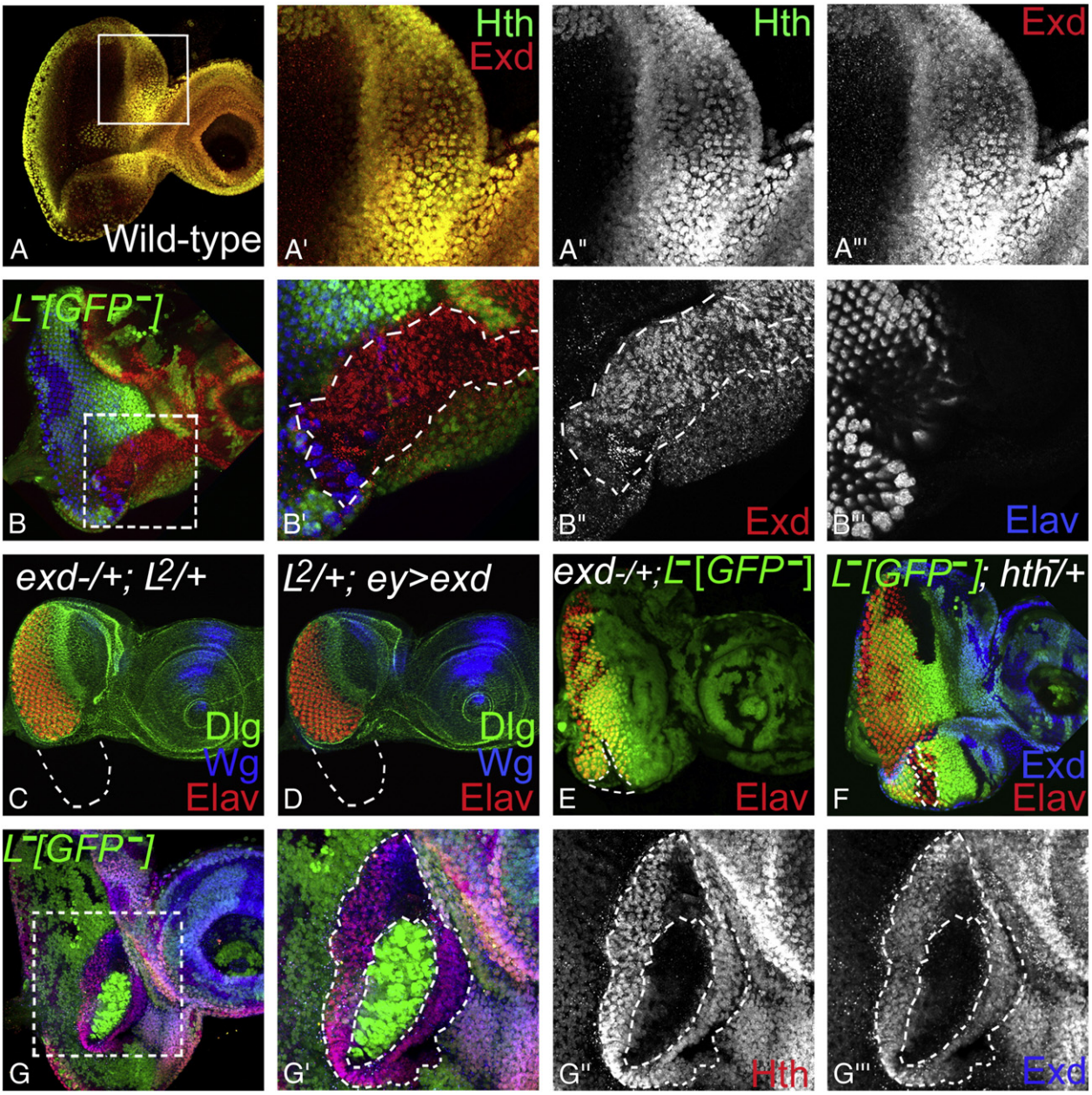

Fig. 6. Exd may not be critical for L and Hth interaction in the ventral eye. (A–A″′) Wild-type expression of (A′) Hth and (A″) Exd in the eye imaginal disc is shown. Exd is nuclear only anterior to the MF where Hth is expressed. In contrast, Exd is cytoplasmic in the eye field where Hth is not present. (B–B″) Loss-of-function clone of L^{rev} in the eye imaginal disc resulted in the loss-of-ventral-eye along with ectopic nuclear localization of Exd in the eye field. Dotted outline marks the area of the ventral clone magnified (B′–B″′) to show the Exd localization and Elav expression. (C) Reducing *exd* levels to half in the $L^2/+$ mutant background does not affect the loss-of-ventral-eye phenotype. (D) Overexpression of *exd* in *L* mutant background (L^2*; ey>exd)* has no effect on the ventral eye loss phenotype. (E) Reducing *exd* level does not affect the L^{rev} loss-of-function phenotype. (F) Loss-of-function clones of L^{rev} in the *hth* heterozygous background do not show suppression of eye and no ectopic nuclear localization of Exd. (G–G″) Loss-of-function clone of L^{rev} in the eye disc results in the ectopic nuclear localization of Exd accompanied with the ectopic induction of Hth. Note that *hth* is not expressed in the eye disc posterior to the MF. Dotted outline in G marks the area of the ventral clone magnified (G′–G″′) to show the Exd and Hth localization.

each other or Exd is not a rate-limiting factor for the Hth function in the *L* mutant background. Therefore, in order to understand the nuclear localization of Exd in *L* mutant clones in the ventral eye, we tested the expression of both Hth and Exd. We found that in loss-of-function clones of *L* in the ventral eye, both Hth and Exd were ectopically localized in the nucleus (Figs. 6G–G″′). Note that 6G′–G″′ are the magnified views of the clone. Thus, Exd nuclear localization in the *L* loss-of-function clones may be due to ectopic induction of Hth. It is known that Hth can form a complex with Exd and drive the hetero-dimer complex to the nucleus. We tested this hypothesis by making the *L* loss-of-function clones in a heterozygous background of *hth* null allele ($L^{-/-}$; $hth^{-/+}$) and observed that there was no ventral eye loss and Exd was no longer nuclear in these clones (Fig. 6F). Thus, *L* interaction with *hth* may not solely depend on nuclear Exd localization.

Discussion

During organogenesis, axial patterning plays a crucial role in transition of a monolayer of primordium cells into a three-dimensional organ. One of the interesting facets of patterning is constant refinement of a large multipotent developing field into smaller fields by progressive restriction of cell fates. These smaller subfields within a developing field are called compartments (Curtiss et al., 2002;

Dahmann et al., 2011). However, there are some interesting questions pertaining to this complex process of sequential restriction of cell fates. For example (i) how are the new compartment boundaries laid within a developing field comprising of a homogenous cell population? (ii) What decides where the boundary will be established within a single or two adjoining developing fields? *Drosophila* eye serves as an excellent model to address these questions of positional fate restrictions as the genetic circuitry involved in retinal determination, axis determination and genes involved in negative regulation of eye fate are known. In this study, we investigated the mechanism responsible for generating the boundary between the developing eyes versus the head field on the ventral eye margin. Interestingly, both head cuticle and eye field are generated from the same eye-antennal imaginal disc, which begins as a homogenous group of cells in the eye primordium. Thus, further assignment of the developmental fates within the eye field by differential regulation of gene expression, will result in delineation of eye versus head fate (Kenyon et al., 2003; Kumar and Moses, 2001). Although the genes involved in eye versus head fate are known but how does their interaction fine tune the boundary between the head versus eye fields is not clear.

In *Drosophila* eye, DV patterning, an essential component of axial growth, is the first lineage restriction event (Singh et al., 2005b; Singh and Choi, 2003). DV patterning results in the generation of dorsal and ventral compartments in the eye (Dominguez and Casares, 2005; Singh et al., 2005b). In *Drosophila*, ventral is the default state of early eye primordium. The default ventral eye fate depends on the function of the *L* gene (Singh et al., 2005b; Singh and Choi, 2003). The homogenous group of cells of early eye primordium with ventral fate gets divided into two different dorsal and ventral fates after the onset of expression of dorsal selector *pnr*. The boundary between the dorsal and ventral compartments is crucial for the growth of eye as an organ.

There is also a boundary between the eye field and the prospective head cuticle. Previously, we have shown that the boundary between developing eye field and the head cuticle on the dorsal margin is regulated by *pnr* gene function (Oros et al., 2010). However, *pnr* is not expressed in the ventral eye. Therefore, a different genetic mechanism might be in place to generate the boundary between eye and the head cuticle on the ventral margin. Here, we have focused on the question pertaining to delineation of the boundary between the head cuticle and the developing eye field on the ventral margin (Fig. 7).

The *Drosophila* eye primordium begins from the ventral fate on which the dorsal eye fate is established. *L* plays a role in ventral eye development, growth and survival. Loss-of-function of *L* results in preferential loss of ventral eye (Figs. 1, 2). We found that *hth*, a modifier of *L* mutant phenotype in the ventral eye (Fig. 1), exhibits ventral specific function. Loss-of-function of *hth* results in enlargement of the eye on the ventral margin of the developing eye field (Fig. 2). Thus, *L* and *hth* exhibit complementary loss-of-function phenotype, and may act antagonistic to each other (Fig. 3). This conclusion is based on (i) ectopic induction of Hth in the loss-of-function clones of *L*, (ii) reducing *hth* gene function, either by a classical mutant approach or by using dominant negative strategy, rescues the *L* mutant phenotype of loss of ventral eye (Fig. 3), and (iii) enhancing *hth* gene function enhances the *L* mutant phenotype of loss-of ventral eye to a "No-eye" (Fig. 1).

Optimum levels of L and hth define the boundary of eye and head on ventral margin

Our studies show that the fine tuning of optimal levels of L and Hth defines the boundary of the eye on the ventral margin. Under wild-type conditions, *L* promotes ventral eye development (Chern and Choi, 2002; Singh et al., 2005b; Singh and Choi, 2003) whereas *hth* promotes the head cuticle fate on the ventral eye margin (Pai et al., 1998; Pichaud and Casares, 2000). However, there is no information available about

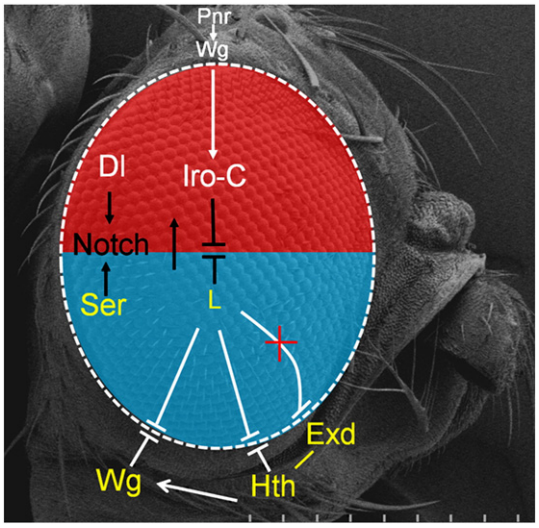

Fig. 7. Antagonistic interactions of *L* with *hth* define the boundary between the head cuticle and the developing eye field on the ventral margin. *L*, a gene required for ventral eye development, interacts antagonistically with the dorsal eye selector *pnr* to define the equator (Singh et al., 2005a). Equator is the boundary between the dorsal and ventral compartments in the eye. This study shows that the boundary between the eye field on the ventral margin and the head cuticle depends on the antagonistic interaction between *L* and *hth*. The fine tuning of the levels of *L* and *hth* is crucial to define the boundary of the eye field on ventral margin. Interestingly, *exd* may not be critical for the antagonistic interactions between *L* and *hth* in the ventral eye. Exd forms a heterodimer with Hth and resultant Hth–Exd dimer is transported to nucleus. It is known that Hth–Exd dimer present in nucleus suppresses the eye fate.

their mutual interaction. Our study demonstrates that *L* acts antagonistically to *hth* (Figs. 3, 7). Therefore, the size of the eye field in the ventral domain is an outcome of fine tuning of balance in *L* and *hth* levels. If the balance shifts in favor of *hth* (*L* mutant background), it results in the loss-of-ventral-eye whereas in converse situation where balance shifts away from *hth* (*hth* mutant background), it results in the enlargement of the ventral eye domain (Fig. 7).

L promotes ventral eye development by suppressing Wg signaling (Singh et al., 2006). Wg is known to act as a negative regulator of eye (Pichaud and Casares, 2000; Treisman and Rubin, 1995). Ectopic upregulation of Wg signaling in the *L* mutant background results in the loss of ventral eye (Singh et al., 2006). However, it is not clear how L regulates Wg signaling to regulate ventral eye development. Wg is expressed strongly in the dorsal eye margin as compared to the ventral eye margin. Removal of Wg in the dorsal eye results in ectopic furrow with similar results in ventral, however with less penetrance (Pichaud and Casares, 2000; Treisman and Rubin, 1995). Wg regulation in the dorsal and the ventral eye is different. In the dorsal eye, Wg acts downstream of Pnr (Maurel-Zaffran and Treisman, 2000). In the ventral eye, Hth maintains Wg, and they act in a positive feedback loop to suppress the eye fate (Pichaud and Casares, 2000; Singh et al., 2005b). We have found that *L* and *hth* interact antagonistically to each other. Therefore, the genetic interaction of L and Wg in the ventral eye (Singh et al., 2006) may be mediated through Hth. Hth, Teashirt (Tsh) and PAX-6 homolog Eyeless (Ey) are coexpressed in a region anterior to the morphogenetic furrow and their complex is responsible for cell proliferation (Bessa et al., 2002; Lopes and Casares, 2010). We have earlier shown that *tsh* and *L* do not interact (Singh et al., 2004). Furthermore, *L* may act downstream of *ey* (Singh unpublished data). Therefore, in light of these evidences L and Hth interaction may be exclusive.

L interacts with MH domain containing alternative spliced variant of Hth

Hth is known to form two different alternative spliced variants (Glazov et al., 2005; Noro et al., 2006). Our studies on domain

requirement suggested that evolutionarily conserved MH domain of Hth is crucial for its interaction with *L* mutant phenotype (Fig. 4). We found that misexpression of transgene encoding truncated Hth protein lacking MH (Hth ΔMH) domain does not affect the *L* mutant phenotype of ventral eye loss whereas the misexpression of transgene encoding Hth protein lacking HD ($Hth^{\Delta HD}$) enhances the *L* mutant phenotype of loss of ventral eye to "no-eye". In fact, the effect of misexpression of $Hth^{\Delta HD}$ was similar to Hth^{FL} on the *L* mutant eye phenotype (Fig. 4). These results suggested that MH domain of Hth is crucial for its interaction with L. Interestingly; we found strong interaction of *L* with $hth^{100\text{-}1}$ (HD-less), an alternative spliced variant of Hth, which does not have a homeodomain. Since MH domain of Hth is required for its interaction with Exd, we tested interaction of L with Exd.

Exd may not be a critical factor for L and Hth interaction in the ventral eye

Hth is required for nuclear localization of Exd. Exd forms a heterodimer with Hth, and Hth–Exd heterodimer is then shuttled to the nucleus to carry out its function. Exd is functional only when it is present in the nucleus (Aspland and White, 1997; Mann and Abu-Shaar, 1996). Hth is required for Exd nuclear localization and function whereas Hth requires Exd for its stability. It has been shown that some of the functions require both Hth–Exd whereas some only require nuclear Exd. Both Hth and Exd loss-of-function show similar phenotype in the eye thereby suggesting both are required for eye development. Therefore, we tested whether *L* interacts with *hth* or with Hth–Exd complex to define the ventral eye margin. Interestingly, we found that L–Hth interaction to define the margin of the ventral eye may work by a novel mechanism which is not critically dependent of Exd (Fig. 6). Our conclusions were supported by the results from our experiment where *L* mutant phenotype in the ventral eye was rescued by misexpression of dominant negative Hth (hth^{ENR}). It has been shown that dominant negative Hth (Hth^{ENR}) does not interfere with the nuclear localization of Exd and that it is capable of driving Exd into the nucleus (Inbal et al., 2001). Thus, nuclear localization of Exd is not sufficient to mediate the Hth function in the *L* mutant background. Furthermore, genetic epistatic analysis of *L* and *exd* showed that they do not interact (Fig. 6). These findings suggest that genetic interaction between L and Hth in the ventral eye is independent of Exd or that Exd is not a rate-limiting factor.

Therefore, our results suggest that ventral eye development gene *L* antagonistically interacts with *hth*, a negative regulator of eye to define the ventral eye margin (Fig. 7). Surprisingly, *L* and *hth* are expressed in both the dorsal and the ventral half of the eye. However, their functional domain (Fig. 2) as well as their antagonistic interaction is restricted only to the ventral half of the eye. It is possible that either the interaction between L and Hth is not direct or there is a factor in the dorsal domain that prevents the interaction of L and Hth in the dorsal half of the eye. It is possible that dorsal selector *pnr*, which establishes the dorsal fate over the default ventral eye fate, might be that factor. It is reported that loss-of-function of *pnr* results in enlargement of the dorsal eye (Maurel-Zaffran and Treisman, 2000; Oros et al., 2010).

L is an ortholog of PRAS40 (Oshiro et al., 2007; Vander Haar et al., 2007; Wang and Huang, 2009) and *hth* is a *Drosophila* homolog of MEIS1 that plays an important role in vertebrate eye development (Bessa et al., 2008; Mann and Abu-Shaar, 1996; Moskow et al., 1995; Pai et al., 1998; Rieckhof et al., 1997). Thus, there is a strong possibility that similar regulatory interactions between L and Hth may occur in the higher organisms that may have implications on the development of field boundaries.

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